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Determinants of 4-aminobiphenyl-DNA adducts in bladder cancer biopsies

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Exposure to 4-aminobiphenyl (4-ABP) is an important determinant of urinary bladder cancer in humans. We have analyzed by gas chromatography–mass spectrometry the DNA adducts of 4-ABP in 75 bladder cancer biopsies. The purpose was to understand whether smoking, *N*-acetyltransferase 2 (*NAT2*) polymorphism, diet or tumor grade were determinants of 4-ABP-DNA levels. 4-ABP-DNA adducts were above the detection limit of 0.1 fmol/μg DNA for 37/75 patients. Overall the level of adducts was 2.7 ± 0.7 (mean ± SE) fmol/μg DNA (86 ± 22 adducts/10^8 normal nucleotides, mean ± SE). A strong association with grade was observed. In the group of patients with detectable 4-ABP-DNA adducts the odds ratio for having a tumor grade of 2 or 3 was respectively 4.3 (95% CI 0.8–21.9) and 6 (1.3–27.5), compared with grade 1. A non-statistically significant association was found between adduct levels and the deduced slow acetylator phenotype in grades 2 and 3. The intake of fruit and vegetables produced a lower frequency of detectable adducts, though the association was not statistically significant. Detectable 4-ABP-DNA adducts were clearly associated with current smoking in higher tumor grades (grade 3 versus grades 1 + 2, odds ratios 10.4; 95% CI 1.7–63.1). Overall, our findings indicate that higher levels of DNA adducts characterize more invasive tumors (higher tumor grades). This seems to be facilitated by smoking and contrasted by the intake of fruit and vegetables.

Introduction

Studies in humans and in experimental animals indicate that carcinogen-DNA adducts are mechanistically relevant to cancer (1,2). No study is available on the direct relationship between adducts at any organ site and cancer risk at that site. Indirect evidence, however, suggests that white blood cell (WBC) adducts predict cancer onset. Retrospective studies on WBC aromatic-DNA adducts, in fact, have shown that cancer cases have higher levels of adducts than non-cancer controls, after adjustment for relevant exposures such as smoking (3–6). Also a prospective study is now available, with similar results (7). Thus, DNA adducts, besides being markers of a biologically active dose, can be considered markers of susceptibility to cancer (8). In addition, WBC aromatic-DNA adducts were related to the levels of organ-site adducts in the case of lung cancer (9), while hemoglobin adducts of 4-aminobiphenyl (4-ABP) were related to 4-ABP-DNA adducts in exfoliated urothelial cells from the urine of healthy smokers (10).

Exposure to 4-ABP, a constituent of tobacco smoke belonging to the chemical class of arylamines, in occupational and environmental settings has been an important determinant of urinary bladder cancer in humans (11). 4-ABP requires metabolic activation to reactive electrophiles, whose binding to DNA is thought to be essential for carcinogenic effects to occur. Hepatic cytochrome P450 1A2 (CYP1A2) has been proposed to mediate the formation of *N*-hydroxylamine, believed to be the first critical step in the bioactivation of 4-ABP (12). *N*-Hydroxylation of 4-ABP competes with the activity of *N*-acetyltransferases. Whereas *N*-acetylation of aromatic amines in general represents a detoxifying process, *O*-acetylation of *N*-hydroxylated amine is regarded as an activation step (13). The enzymatic activities of CYP1A2 and of the two *N*-acetyltransferases, NAT1 and NAT2, involved in 4-ABP activation/detoxification show wide interindividual variation thus modifying the bladder cancer risk associated to 4-ABP exposure (13,14). NAT2 polymorphism segregates the human population into phenotypically rapid and slow acetylators; epidemiological evidence suggests that slow acetylators are at higher risk of bladder cancer (15). This generates the hypothesis that the acetylation polymorphism might modify the formation of adducts to the target DNA, as well as to surrogate DNA and blood proteins, however only a few studies have investigated the effect of *NAT1* and/or *NAT2* genotype or phenotype on the modulation of the specific 4-ABP adducts to hemoglobin or DNA (16–22). Dietary fruits and vegetables may play a preventive role for a number of cancers, though epidemiological studies on bladder cancer incidence show contradictory results (23,24). These foods are a source of vitamins and phytochemicals that may have anticancer activities. Proposed mechanisms include antioxidant properties, inhibition of carcinogen metabolizing enzymes and inhibition of tumor cell proliferation (25). These activities might modulate the formation of DNA adducts. We have previously shown in a bladder cancer case-control study that WBC aromatic-DNA adducts were lower in subjects with elevated intake of fruit and vegetables (4).

We have conducted a study on 75 bladder cancer biopsies in which we have measured the DNA adducts of 4-ABP. The purpose of the present paper was to investigate whether smoking, *NAT2* polymorphism, diet or tumor grade are determinants of 4-ABP-DNA adduct levels in bladder cancer biopsies.

Abbreviations: 4-ABP, 4-aminobiphenyl; CYP1A2, cytochrome P450 1A2; GC-NICI-MS, gas chromatography-negative ion chemical ionization-mass spectrometry; NAT, *N*-acetyltransferase; OR, odds ratios; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*-pyridine; WBC, white blood cells.

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Patients and methods

A total of 114 incident (newly diagnosed) cases of bladder cancer with a histologically confirmed diagnosis, living in the Torino Metropolitan Area, were recruited. They were Caucasian men, aged 40–74, treated in the Urology Departments of the Gradenigo and S. Giovanni hospitals in 1994–1995 and included 52 patients previously described (22). The identification of cases occurred through daily contacts between a trained interviewer and the Urology Departments. Histological confirmation was obtained from the Pathology Departments. Patients were interviewed using a questionnaire including a detailed history of tobacco smoking, a simplified 24 h recall for dietary habits, drug use and occupational history. A limitation of the interview was that the dietary habits of the patients had changed because of symptoms of the disease or hospitalization, thus artificially increasing the number of those who had not consumed fruit and vegetables the day before interview.

After informed consent, blood was drawn (40 ml) and immediately centrifuged. Buffy coat, red blood cells and plasma were separated and stored at –80°C. Biopsies were collected; a small piece was kept and stored at –80°C, only when the biopsy was sufficiently large to allow diagnosis. Biopsies were examined by a single pathologist. For each histological sample, the borders of the neoplastic area were carefully drawn with a pen on the hematoxylin–eosin slides, under microscopical control. The material was then cut with a razor blade. A further histological section was obtained from the modified blocks, in order to check that non-neoplastic cells had been eliminated. Carcinomas were divided into three grades according to the WHO classification.

DNA was extracted from bladder biopsies using Nucleobond AX cartridges (Machery-Nagel Gmbh KG, Düren, Germany) according to the manufacturer's instructions, and analyzed for 4-ABP-DNA adducts.

Quantitation of 4-ABP-DNA adducts was performed at the 'Mario Negri' Institute by alkaline hydrolysis and gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) according to the method published by Lin *et al.* (26) with minor modifications. Briefly, aliquots of about 100 µg DNA were spiked with a known amount of deuterated 4-ABP used as internal standard, and then hydrolyzed in 0.05 N NaOH at 100°C overnight. The liberated 4-ABP was extracted with hexane and derivatized using pentafluoropropionic anhydride in trimethylamine for 30 min at room temperature, prior to GC-NICI-MS.

Also DNA adducts in white blood cells have been measured by the ^{32}P-postlabelling technique, as described elsewhere (4). *NAT2* genotyping was done on leukocyte DNA according to Bell *et al.* (27) with the modifications previously described (4). Content in phenolics and antimutagenic activity of plasma Bond-Elut C-18 extracts were measured as described (28). All the phases of the study till the final statistical analyses were blind.

We have computed means and medians of DNA adducts. Analysis of variance and non-parametric methods were used for significance testing (29). The median of 4-ABP-DNA adducts was used to compute odds ratios (OR) and their 95% confidence intervals. When relevant, multivariate analyses (multiple regression) were performed with the SAS package for personal computer. All OR are adjusted by age.

Table I. Adducts of 4-ABP-DNA in 71 bladder cancer biopsies: distribution by tumor grade

Adduct levels	Tumor grade			Total
	1	2	3	
	No. of patients (%)			
Non-detectable	10 (29)	17 (46)	7 (32)	34 (100)
<1.05 fmol/µg DNA[a]	2 (11)	8 (22)	8 (36)	18 (100)
>1.05 fmol/µg DNA	0 (-)	12 (32)	7 (32)	19 (100)
Total	12	37	22	71
Odds ratios	**No. of patients with level of adducts above/below limit of detection**			**Total**
	2/10	20/17	15/7	37/34
Crude	1	5.9	10.7	
(CI)[b]	(reference)	(1.1–30.6)	(1.8–62.5)	
Age-adjusted	1	4.3	6.0	
(CI)	(reference)	(0.8–21.9)	(1.3–27.5)	

[a]Median value of detectable 4-ABP-DNA adducts.
[b]CI: 95% confidence interval.

Table II. Frequency of 4-ABP-DNA adducts in 71 bladder cancer biopsies: distribution by tumor grade and NAT2 deduced phenotype. OR = age-adjusted odds ratio; CI = 95% confidence interval

NAT2 deduced phenotype	Tumor grade 1	2	3	Total[a]
	No. of patients with level of adducts above/below limit of detection			
Rapid	0/1	6/7	4/3	10/11
Slow	2/9	13/8	11/4	26/21
OR slow vs. rapid	–	1.8	1.7	
	(CI)	(0.4–7.5)	(0.2–15.8)	

[a]Genotype missing for three subjects.

Results

The analyses of 4-ABP adducts are based on 75 biopsies and include 45 biopsies previously described (22); for 39/114 biopsies the amount of DNA was insufficient for analysis (<30 µg). The mean age of these patients was 63.4 ± 7.5 (mean ± SD, range 40–74).

The detection limit of the method for 4-ABP-DNA adducts quantitation was 0.1 fmol/µg DNA using 30–100 µg DNA. However, in a few instances, when more DNA was available (i.e. large biopsies), we have been able to detect adduct levels below this limit.

4-ABP-DNA adducts were present in 37/75 patients, ranging between undetectable and 35.8 fmol/µg DNA. Overall adduct level was 2.7 ± 0.7 (mean ± SE) fmol/µg DNA, corresponding to 86 ± 22 adducts/10^8 normal nucleotides (mean ± SE). To compute these means, one half of the detection limit was used for adduct levels below the detection limit.

Table I shows the distribution by tumor grade and by the presence/absence of a detectable level of adducts. Tumor grade was not available for four patients, thus statistical analyses refer only to 71 cases. A strong association with grade was evident, later grades showing detectable adducts more frequently. The risk for having a tumor grade of 2 or 3 was respectively 4.3 and 6 times higher in patients with detectable 4-ABP-DNA adducts in their bladder biopsies than in those with no detectable adducts. Patients who were NAT2 slow acetylators had more frequently detectable adducts if they had grade 2 or 3 tumors (Table II).

Table III reports the distribution of detectable 4-ABP-DNA by fruit and vegetable intake. The frequency of detectable adducts was lower in subjects who consumed one or more portions of fruit and vegetables per day than in those who did not, but the differences were not statistically significant. Only eight out of the 75 bladder cancer patients were never smokers, the majority being current smokers (46/75), smoking 21 ± 1.5 cigarettes/day (mean ± SD) or former (17/75) smokers. Detectable 4-ABP-DNA adducts were present in ~50% of the patients regardless of their smoking habits, however they were clearly associated with current smoking (but not with former smoking), in higher tumor grades (Table IV). Smokers of air-cured tobacco with detectable adducts had a higher risk of

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Table III. Frequency and levels of 4-ABP-DNA adducts in 71 bladder cancer biopsies: distribution by tumor grade and intake of fruit and vegetables. OR = age-adjusted odds ratio; CI = 95% confidence interval

Fruit and vegetable intake	No. of individuals with various adduct levels:			Total
No. of portions	nd[a]	<1.05 fmol/μg DNA[b]	<1.05 fmol/μg DNA[b]	
None	11	8	9	28
1–2	16	8	7	31
3 or more	7	2	3	12
OR 1 + vs. 0	–	0.6	0.6	71
(CI)	–	(0.2–2.1)	(0.2–2.1)	
Adduct levels	Adduct levels (fmol/μg DNA) for various portions of fruit and vegetable intakes:			All
	0	1–2	3 or more	
Mean	4.2	1.9	1.3	2.7
Standard error	1.6	0.8	0.7	0.7
75° percentile	5.6	0.5	1.05	2.02

[a]nd = non-detectable.
[b]Median value of detectable 4-ABP-DNA adducts.

Table IV. Frequency of 4-ABP-DNA adducts in 71 bladder cancer biopsies: distribution by tumor grade and smoking habits. OR = age-adjusted odds ratio; CI = 95% confidence interval

Smoking habits	Tumor grade		Total	OR (CI)[a]
	1 + 2	3		
	No. of patients with level of adducts above/below limit of detection			
Never smoker	4/1	1/2	5/3	–
Ex-smoker	4/5	4/4	9/8	0.7 (0.15–6.4)
Current smoker	14/21	10/1	24/22	10.4 (1.7–63.1)
Total	22/27	14/7	37/34	
Type of tobacco	Tumor grade (smokers only)			
	1 + 2	3		
	No. of patients with level of adducts above/below limit of detection			
Flue-cured	2/3	1/0	3/3	–
Mixed	13/14	9/3	22/17	2.5 (0.5–13.2)
Air-cured	3/9	4/2	7/11	5.7 (0.9–35)
Total	18/26	14/5	32/31	

[a]Grade 3 versus grades 1 + 2.

having a grade 3 tumor compared with flue-cured tobacco smokers (Table IV).

The interaction between smoking habits, fruit and vegetables intake, and tumor grade affected 4-ABP-DNA levels. Adduct values (mean ± SE fmol/μg DNA) were ≤0.1 (n = 1), 2.6 ± 0.9 (n = 16), and 6.8 ± 3.7 (n = 11) for tumor grades 1, 2, and 3 respectively, in patients who did not consume fruit and vegetables the day before the interview. Interestingly, in grade 3 the intake of fruit and vegetables was clearly associated with a reduced concentration of adducts (6.8 ± 3.7 fmol/μg DNA (n = 11), 4.6 ± 2.9, n = 7, and 0.55 ± 0.29, n = 4, for patients consuming none, 1–2 portions, and >3 portions of fruit and vegetables respectively). Out of 12 subjects with a grade 1 tumor, 10 had non-detectable levels of adducts, and two had levels lower than the median. Of the 10 patients with no detectable adduct levels, nine had eaten fruit or vegetable in the previous 24 h. None of the differences was statistically significant.

Current smokers had adduct levels of 5.3 ± 2.3 fmol/μg DNA (n = 18) if they had not consumed fruit and vegetables the day before the interview. These values dropped to 0.63 ± 0.4 (n = 20) and 1.84 ± 1.1 (n = 8) for consumers of 1–2 portions and ≥3 portions of fruit and vegetables respectively. Again, these differences did not reach statistical significance.

To better understand the mechanisms by which fruit and vegetables might reduce the level of adducts we have investigated the relationship between the content in phenolics and the antimutagenicity of plasma extracts and 4-ABP-DNA adducts in bladder tumor biopsies. The content of phenolics in plasma extracts was 8.92 ± 2.38 ng/μl plasma equivalent (mean ± SD) and antimutagenicity of plasma extracts expressed as decrease in revertants per μl plasma equivalent was 19 ± 6.52 (mean ± SD). Among the subjects who did not consume fruit or vegetables, phenolics were 9.4 and antimutagenicity was 20.6; among those consuming 1–2 portions, they were 9 and 17.4, respectively, and among consumers of three or more portions they were 7.4 and 19. None of these differences was statistically significant.

If we consider only detectable 4-ABP-DNA adducts (≥0.1 fmol/μg DNA), the correlation coefficient between phenolics and the logarithm of adducts is –0.41 (P = 0.02), while the coefficient for antimutagenicity is –0.30 (P = 0.09). In a multivariate model with log(adducts) as the dependent variable, regression coefficients were 0.12 for age (P = 0.04), –0.07 for phenolics (P = 0.03), and –0.03 for antimutagenicity (P = 0.02), after adjustment for tumor grade. The median adduct level was 3.25 fmol/μg DNA (75° percentile 5.08) for a level of phenolics lower than the median, versus 2.29 fmol/μg DNA (75° percentile 0.99) for a level of phenolics greater than the median. Very similar values were observed in subjects with levels of antimutagenicity below and above the median.

No correlation was found between bulky DNA adducts measured by ^{32}P-postlabelling in WBC reported in a previous study (30) and 4-ABP-DNA adducts analyzed in this study. After log transformation of both variables, correlation coefficients were 0.031 (P = 0.78) when considering all samples, and 0.096 (P = 0.57) when considering only samples with detectable levels of 4-ABP adducts.

Discussion

In a case–control study nested in the prospective Physicians Health Study cohort, DNA adducts measured by ^{32}P-postlabelling in WBC from current smokers were predictive of the cancer outcome (7). In that study the measurements of adducts were made in blood samples that were collected years before cancer onset, thus ruling out the possibility that the higher adduct levels were due to the metabolic changes associated with an already existing cancer. We do not have similar evidence on the predictive value of organ-site adducts in terms of cancer risk, this being a case-only study. However, indirect evidence is available, since WBC bulky adducts have been shown to correlate with adducts measured at the organ level (9). At variance with this we observed that 4-ABP-DNA

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adducts in bladder biopsies were not correlated with WBC bulky adducts previously analyzed in a case-control study on bladder cancer from which the present case series is derived (4). This comes with no surprise owing to the different methodologies applied for the analysis of DNA adducts in WBC and in bladder biopsy. ^{32}P-Postlabelling, used for WBC, detects total bulky adducts produced by a number of environmental carcinogens, whereas GC-NICI-MS, used for bladder cancer biopsies, measures specifically only adducts resulting from 4-ABP exposure. These adducts are only a fraction of those measured by ^{32}P-postlabelling (26). Also the bioavailability of 4-ABP and the kinetics of DNA adduct formation and removal within the bladder tumor tissue might be different from that of leukocytes.

Patients who have been diagnosed bladder cancer with an early grade (low invasiveness) have very low levels of adducts, irrespective of smoking habits, thus confirming a preliminary observation on a smaller group of bladder cancer patients (22). This is in agreement with the study by Curigliano *et al.* showing a non-statistically significant grade-related increase in mean relative staining intensity for 4-ABP-DNA adducts analyzed in bladder cancer biopsies using an immunohistochemical method (31). High-grade bladder tumors are characterized by aneuploidy with increased S-phase fractions and cell proliferation (32), thus providing an increased number of targets for 4-ABP reactive metabolites.

DNA-adduct formation is regarded as an initial step in the multistage process of carcinogenesis. The observation of a grade-related presence of 4-ABP-DNA adducts, however, suggests that 4-ABP might also be involved in late events of the carcinogenic process. In support to this is the observation that 4-ABP can cause both tumorigenic transformation and neoplastic progression in *in vitro/in vivo* experimental models using human uroepithelial cell lines inoculated into athymic nude mice after exposure to 4-ABP (33).

We have no information on the persistence of DNA adducts in humans, but they probably indicate a cumulative damage resulting from relatively recent or continuous exposure, such as that derived from smoking. The higher frequency of detectable adducts in grade 3 tumors suggests this is the result of a joint effect of different determinants. Several inherited or lifestyle factors are known to modulate adduct formation, accumulation and removal. They include smoking habit, fruit and vegetables intake, and genetic polymorphisms of the enzymes involved in carcinogen activation/detoxification or DNA repair (8).

The present study shows that patients with higher tumor grades were associated with low fruit and vegetable intake, NAT2 slow phenotype, current smoking and high adduct levels. The intake of fruit and vegetables was associated with reduced adduct levels in current smokers (but not clearly in ex-smokers).

We have previously shown that the level of WBC aromatic-DNA adducts decreased with increasing levels of fruit and vegetable consumption; in addition, the association between the case–control status and the level of adducts (below or above the median value) was stronger in the subjects who consumed less than two portions of vegetables per day than in heavy consumers (4). Also in a study of healthy volunteers, conducted in the context of the EPIC Italian cohort, an inverse association between the intake of fruit and vegetables and adduct levels was found (34). Altogether these data are in good agreement with the epidemiological evidence of a protective role of fruit and vegetables on bladder carcinogenesis (23,24).

A limitation of the present study was that the dietary habits of the patients had changed because of symptoms of the disease or hospitalization, thus artificially increasing the number of those who had not consumed fruit and vegetables the day before interview. Therefore, our observation requires confirmation in the context of prospective investigations.

The role of fruit and vegetables seems to be attributable to the presence of phenolics in plasma and to their antimutagenic activity, but this is still a hypothesis. In agreement with previous studies (28), the present one lends support to the hypothesis of a protective role of phenolics, through an inverse association between plasma phenolic levels and the level of DNA adducts in bladder biopsies. Dietary phenolics include flavones, flavonols and isoflavones which are know to act as antioxidants and to interfere with the metabolic activation/detoxification of chemical carcinogens (35).

The NAT2 slow phenotype has been reported to be associated with higher levels of aromatic amine-DNA adducts in bladder biopsies (21). In line with this, our results show a higher frequency of 4-ABP-DNA adducts in slow acetylators, but only in grade 2 and 3 tumors.

The presence of high levels of adducts in smokers is consistent with the notion that CYP1A2 involved in 4-ABP metabolism is highly induced by smoke (36,37). The higher the adduct levels, the higher the probability that a mutation in relevant genes could occur. We have previously shown that mutations in the tumor suppressor gene *TP53* were more frequent in grade 3 tumors than in less invasive ones (22). Expression of wild-type p53 is required for the efficient global genomic nucleotide excision repair of UV-induced cyclobutane pyrimidine dimers or bulky adducts (38,39). Using human papillary transitional cell carcinoma cell lines, Torino *et al.* reported that p53 is involved in the repair of 4-ABP-DNA adducts, the rate of repair being slower in cells expressing the mutant form of p53 (40). The resulting accumulation of DNA adducts and accompanying mutations might increase genomic instability. In general, an increased mutation rate is regarded as a growth disadvantage for the cell, unless there is a selection pressure exerted, for instance, by specific carcinogens, that favours cell growth (41,42). In support to this, it has been shown that cell exposure to 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a bulky adduct forming carcinogen, causes chromosomal instability. Moreover, cells made resistant to PhIP also show chromosomal instability, indicating a mechanistic link between these two phenotypes and suggesting that cells with defects in DNA repair or mitotic checkpoints or other still unknown defects might be selected after the exposure to specific carcinogens (42).

Overall, a possible interpretation of our findings is that malignant clones undergo selection in such a way that cells carrying higher levels of DNA adducts characterize more invasive tumors (higher tumor grades). Such clonal selection is facilitated by smoking and contrasted by the intake of fruit and vegetables. There is some evidence to support this: (a) adducts were very low in tumors with limited aggressiveness (low grade); (b) smoking was associated with both adduct levels and grade; (c) virtually all grade 1 tumors occurred in subjects eating fruit and vegetables, and showed non-detectable levels of adducts.

The hypothesis of clonal selection is compatible with recent theories of carcinogenesis that hypothesize that cancer arises

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as a consequence of a number of mutational steps in key genes, with clonal expansion of intermediate cells. 'Darwinian selection' has been proposed as a plausible mechanism for carcinogenesis (43). The characteristics of the environment that surrounds the cell, including the presence of smoking-related substances and the lack of protective chemicals contained in fruit and vegetables, are likely to exert a selective pressure in selecting clones with DNA damage.

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